

# Signaling stress? An analysis of phaeomelanin-based plumage color and individual corticosterone levels at two temporal scales in North American barn swallows, *Hirundo rustica erythrogaster*



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## ABSTRACT

Sexually selected traits confer greater reproductive benefits to individuals with more elaborate forms of the signal. However, whether these signals convey reliable information about the physiology underlying trait development remains unknown in many species. The steroid hormone corticosterone (CORT) mediates important physiological and behavioral processes during the vertebrate stress response, and CORT secretion itself can be modulated by melanocortins. Thus, sexually selected melanin-based plumage coloration could function as an honest signal of an individual's ability to respond to stressors. This hypothesis was tested in North American barn swallows, *Hirundo rustica erythrogaster*, where males with darker ventral plumage color exhibit higher phaeomelanin content and are more successful at reproduction. Because reproductive behavior occurs months after plumage signals are developed, we also addressed the potential temporal disconnect of physiological state during trait development and trait advertisement by analyzing three different measurements of CORT levels in adult males during the breeding season (trait advertisement) and in nestling males while they were growing their feathers (trait development). Variation in adult plumage color did not predict baseline or stress-induced CORT, or stress responsiveness. Likewise, there was no relationship between nestling plumage color and any of the CORT measurements, but heavier nestlings had significantly lower baseline CORT. Our finding that a predominantly phaeomelanin-based trait is unrelated to circulating CORT suggests that phaeomelanin and eumelanin signals may convey different physiological information, and highlights the need for further study on the biochemical links between the hypothalamic–pituitary–adrenal (HPA) axis and the production of different melanin-based pigments.

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## Introduction

The theory of sexual selection predicts that variation in morphological or behavioral traits is shaped, in part, by variation in mating success (Andersson, 1994; Darwin, 1859, 1871). Some models of sexual selection (Folstad and Karter, 1992; Zahavi, 1975) indicate that conspecifics derive relevant and reliable information from these variable features of morphology and behavior, such as an individual's fighting ability, health status, or social dominance. Previous research on the potential information conveyed by sexual signals has placed a large emphasis on an individual's physiological state, which is known to impact behavior and fitness, and may also modulate the development and expression of these traits. For example, the steroid hormone testosterone plays a

role in behaviors important to reproduction and has been shown to regulate the elaboration of some types of sexual signals such as promoting plumage size of the melanin-based bib in house sparrows (*Passer domesticus*) (Gonzalez et al., 2001), increasing bioavailability of carotenoids for beak brightness in house finches (McGraw et al., 2006), and controlling the timing of development and brightness of melanin-based ornamental plumage in fairy-wrens (*Malurus cyaneus*) (Peters et al., 2000).

Signal traits have also been proposed to convey information about individual variation in the response to stressors, but these links are not well understood. Glucocorticoids (predominantly corticosterone in avian species; hereafter 'CORT') modulate many physiological and behavioral components important to fitness, including metabolic function and behavioral responses to stressors. CORT secretion occurs in a circadian rhythm to promote mobilization of energy stores through glycogen breakdown, gluconeogenesis, and lipolysis (reviewed in Charmandari et al., 2005). Individuals increase CORT secretion when faced with perturbations, initiating a variety of physiological and behavioral changes that likely increase immediate survival (Seyle, 1937; Wingfield et al., 1998). Given that CORT mediates many important physiological

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processes and behaviors, it is not surprising that a relationship has been found between circulating CORT levels and parental care (Ouyang et al., 2011a), predator avoidance (Cinchy et al., 2011), and survival rates (Blas et al., 2007; Romero and Wikelski, 2010). Moreover, inter-individual variation in both baseline and stress-induced CORT concentrations can be substantial and recent evidence indicates that this variation is correlated with measures of fitness in several avian systems (Bonier et al., 2009 and reviewed in Wingfield, 2003).

Another interesting feature of CORT is that it may be associated with melanin production and pigment deposition – the underlying basis of many morphological signals under sexual selection (e.g. Roulin et al., 2001; Safran and McGraw, 2004; Siefferman and Hill, 2003) – making it a likely candidate in mediating the information content of melanin-based traits (Almasi et al., 2008, 2010; Roulin et al., 2008). Empirical evidence suggests that melanin-stimulating hormone (MSH) exerts pleiotropic effects on many physiological processes by binding to various melanocortin receptors (MCRs 1–5) located throughout the body (reviewed in Ducrest et al., 2008). At the level of the skin  $\alpha$ -MSH binds to MC1Rs in melanocytes to stimulate eumelanin pigment production. Additionally,  $\alpha$ -MSH has been implicated in modulating the stress response by binding to MC4Rs in the hypothalamus and reducing circulating CORT concentrations during a forced swim test (Racca et al., 2005). Due to the suggested link between  $\alpha$ -MSH and eumelanin production, Ducrest et al. (2008) proposed that darker, more eumelanic individuals should exhibit greater “stress resistance” than lighter conspecifics. In support of this hypothesis, the degree of eumelanism is shown to be associated with the ability to return to baseline CORT levels after exposure to a stressor (Almasi et al., 2010) as well as the ability to cope with stressful events by maintaining traits important to survival and reproduction (Almasi et al., 2008, 2012, 2013).

Likewise, there are potential biochemical links between the HPA axis and phaeomelanogenesis that parallels the regulation of stress reactivity and eumelanin production by  $\alpha$ -MSH. Phaeomelanin production in melanocytes is stimulated by the binding of agouti-signaling protein (ASIP) that is locally produced in the skin to MC1Rs. Agouti-related protein (AGRP) is an antagonist of melanocortins that is expressed extensively in the brain where it has been shown to bind to MC4Rs and MC5Rs in the hypothalamus to regulate energy homeostasis (Ollmann et al., 1997) but also stimulates HPA axis activity by increasing CRH, ACTH and CORT secretion (Xiao et al., 2003). Expression of AGRP in chicken skin could serve as an alternative melanocortin antagonist similar to ASIP (Takeuchi et al., 2000). Therefore, AGRP may regulate pigment production whereby individuals with more phaeomelanin deposition would be predicted to have higher circulating glucocorticoids and stress reactivity. Thus, empirical evidence supports the potential for both eumelanin- and phaeomelanin-based traits to provide information about an individual's circulating CORT levels and, likely, their ability to cope with stress.

Despite the potentially important relationship between CORT and melanin-based traits, previous studies have overlooked the complexity of melanin-based signals by classifying these traits as either “eumelanin” or “phaeomelanin”, when in fact many traits are comprised of both pigment types (McGraw et al., 2004). The physiological relationship between HPA axis activity and plumage coloration may be opposing for a trait comprised of both eumelanin and phaeomelanin. Moreover, it remains unclear whether melanin-based traits used as sexual signals convey information about individual variation in corticosterone secretion to a potential mate. The objective of this study is to better elucidate the association between CORT and melanin-based plumage coloration using the North American barn swallow, *Hirundo rustica erythrogaster*, as a study system. Melanin-based plumage color is a sexually selected trait in *H. rustica erythrogaster*, and males with darker ventral plumage color are shown to have greater reproductive success than lighter conspecifics (Safran and McGraw, 2004; Safran et al., 2005). This sexual signal is comprised of both eumelanin and phaeomelanin pigments, and it is the ratio of these two pigments in breast feathers that accurately

predicts male plumage brightness. Specifically, McGraw et al. (2005) showed that although approximately 75% of total melanin content in breast feathers consists of eumelanin, which is fairly consistent between the sexes, it is actually the amount of phaeomelanin deposited into breast feathers that drives variation in plumage coloration among males. Therefore, “darker” male barn swallows have smaller E:P ratios (more phaeomelanin while eumelanin stays fairly constant) which leads to ventral feathers that are browner and redder in color compared to males with lower amounts of phaeomelanin (McGraw et al., 2005).

Based on evidence that suggests several biochemical links between the HPA axis and melanin production pathways, we explored whether variation in the sexually selected melanin-based ventral plumage color of male North American barn swallows predicts individual variation in CORT levels. Specifically, we analyzed natural ranges of variation in both plumage color and CORT secretion to elucidate the relationship between a predominantly phaeomelanin-based plumage trait and CORT using three measurements of total circulating CORT: baseline (collected within 3 min of disturbance), stress-induced (collected after 15 min of handling restraint) and stress responsiveness (the magnitude of change in concentration from baseline to stress-induced CORT). Although the male barn swallow's ventral plumage is comprised of both melanin pigment types, and the proposed relationships between plumage coloration and CORT concentration may be opposing for this complex signal, variation in feather color among individuals is derived primarily from the variation in phaeomelanin content. Given that there is support for a biochemical pathway involving AGRP that is similar to the pleiotropic melanocortin system as described by Ducrest et al. (2008), we propose the *melanin–CORT pleiotropy hypothesis*, which allows us to infer the relationship between phaeomelanin-based signals and individual CORT levels. Accordingly, we predicted that darker, more phaeomelanic males would have higher baseline and stress-induced CORT, and stronger stress responsiveness compared to lighter males that have lower phaeomelanin feather content.

In barn swallows, as in many songbirds, there is a temporal disconnect between the development of plumage signals, which occurs during the non-breeding season, and their use of the signal months later when individuals are often experiencing very different social and ecological conditions. Because both baseline and stress-induced CORT can be variable within individuals (Ouyang et al., 2011b), the predicted relationships between CORT concentrations and feather color may be observed only during the period of feather production. To address this, we measured CORT from individuals in two contexts: adult males during the breeding season (trait advertisement) and nestling males that are actively depositing melanins as they develop feathers (trait development). Like adult plumage color, nestling feather color is highly variable and, importantly, is a strong predictor of their adult color (Hubbard and Safran, unpublished data). Accordingly, an analysis of CORT profiles during nestling plumage development may also be relevant for inferring a role of this hormone in mediating plumage color in adults during molt on non-breeding grounds in the southern hemisphere.

## Methods

### Capture and sampling

This study took place in a population of North American barn swallows in Boulder County, Colorado during their breeding season between May and August 2010. Our investigations were centered on males in two different life stages: breeding adults and nestlings. Adult and nestling blood samples were collected from the brachial vein using a heparinized microhematocrit capillary tube and placed on ice until plasma could be separated from blood by centrifugation. Plasma samples were then stored at  $-70^{\circ}\text{C}$  until assayed. Following a standardized restraint stress protocol, blood samples were collected within 3 min of initial disturbance for baseline CORT (Romero and Reed, 2005) and 15 min after disturbance for stress-induced CORT. Between blood

sample collections mass was recorded and a small sample of breast feathers (~5) was taken from the melanin-based breast patch and stored in a dry, dark envelope until analysis. Our field sampling approach (of approximately five feathers per ventral region) provides a representative estimate of ventral color. This is based on a known correlation between our point samples and an integrated measure of ventral color using standardized photography protocols (Vortman et al., 2011).

We caught 94 adult males across 20 breeding sites between 8 pm and 2 am using either a mist net or by hand off the nest. Adults were banded with an aluminum U.S. Geological Survey (USGS) ring and uniquely marked using a combination of colored plastic leg bands and nontoxic permanent markers on the white spots of tail feathers. The sex of adult barn swallows was initially assessed visually using sexual dimorphism in characteristics such as the darkness of the ventral plumage, tail streamer length (Safran and McGraw, 2004) and the presence or absence of a brood patch, and whenever possible was confirmed by observing parental behavior (e.g. females do most of the incubating). Twelve-day-old nestlings ( $n = 82$  males) were removed from 43 nests by hand across 17 sites between 10 am and 5 pm. Nestlings are not strongly sexually dimorphic, and we therefore assessed the sex of nestlings using molecular tools (see below). All nestlings were banded with aluminum USGS rings and were placed back into the same nest after sample and data collection. Our research protocols were approved by the University of Colorado's IACUC (permit #1004.01), the Colorado Division of Wildlife and the United States Federal Bird-Banding Lab.

#### Corticosterone assays

Total CORT concentrations from baseline and stress-induced plasma samples were quantified using enzyme immunoassay (EIA) kits (catalog number ADI-901-097, Enzo Life Sciences, Plymouth Meeting, PA 19462, USA). Assay procedures were optimized for barn swallow plasma by stripping samples with Norit-activated charcoal and spiking with a known amount of CORT (Wada et al., 2007). Tests across multiple dilution values (1:10, 1:20, 1:40 and 1:80) and concentrations of steroid displacement buffer (0%, 1%, 2%) indicated that a plasma dilution factor of 1:40 with 2% steroid displacement buffer (SDR) was optimal. Both baseline and stress-induced plasma samples from each individual were assayed on the same plate along with a six-standard curve. For adults, standards and samples were run in triplicate with an inter- and intra-assay variation of 9.37% and 12.3%, respectively. For nestlings, standards and samples were run in duplicate with an inter-assay variation of 9.81% and an average intra-assay variation of 9.52%. We switched from running standards and samples in triplicate to duplicate because no significant difference was found in the calculations of the coefficient of variability (CV). All samples were above the detection threshold ( $0.30 \pm 0.09$  ng/ml across plates).

#### Measuring plumage coloration

To objectively quantify plumage color, feathers were collected from the breast region of adults and nestlings, and stored in small envelopes in the dark until measured (Safran and McGraw, 2004). The color of these samples was assessed by measuring plumage brightness using an Ocean Optics USB4000 Spectrometer (Dunedin, FL, USA). Reflectance data were generated relative to a white standard (Ocean Optics WS-1) and a dark standard (all light excluded), and spectra were recorded with the SpectraSuite software package (version 2.0.125, Ocean Optics Inc.). We used average brightness, which was calculated from three separate measurements of the collected breast feathers, as a representative metric of overall ventral plumage color because the three traditional axes of color (hue, chroma, and brightness) were found previously to be highly correlated across the ventral region of individual barn swallows (McGraw et al., 2005; Safran and McGraw, 2004; Hubbard, unpublished data), and brightness is the most variable dimension of

color in this region (Hubbard, unpublished data). Lower brightness scores (% reflectance) indicate plumage color that appears darker, redder, and more saturated when compared to feathers with higher brightness scores.

For consistency in our measures of nestling and adult color, we analyzed breast feathers from the ventral plumage region because this is the only location on the ventral surface where nestling feathers are developed on the day that we collect standardized measures of nestling size. Moreover, all of our measures of plumage color from a transect along the ventral portion of male swallows (throat, breast, belly, and vent) are highly intercorrelated (Safran and McGraw, 2004). Note that we did not find a relationship between adult average throat brightness and any of the CORT measurements analyzed in this study (mixed linear model with site and plasma sampling date as random effects: *baseline CORT*,  $n = 90$ , throat average brightness  $F_{1, 84.5} = 0.23$ ,  $P = 0.63$ ; *mass*  $F_{1, 85.1} = 2.93$ ,  $P = 0.09$ ; *sunset correction*  $F_{1, 78.7} = 2.64$ ,  $P = 0.11$ ; *stress-induced CORT*,  $n = 92$ , throat average brightness  $F_{1, 88} = 0.41$ ,  $P = 0.53$ ; *mass*  $F_{1, 81.6} = 0.00$ ,  $P = 0.96$ ; *sunset correction*  $F_{1, 87.9} = 10.12$ ,  $P = 0.002$ ; *stress responsiveness*,  $n = 92$ , throat average brightness  $F_{1, 86.2} = 0.13$ ,  $P = 0.71$ ; *mass*  $F_{1, 78.1} = 0.40$ ,  $P = 0.53$ ; *sunset correction*  $F_{1, 18.6} = 9.86$ ,  $P = 0.006$ ).

#### Visual color as a proxy for melanin concentrations

The melanin content of breast feathers has been quantitatively analyzed for North American barn swallows. As described in McGraw et al. (2005), objective, quantitative measures of plumage color were shown to be very reliable proxies of the ratio of eumelanin to pheomelanin in barn swallows. Accordingly, in this study we use objective measures of visual color to infer its pigmentary basis. Moreover, although the study on melanin pigments (McGraw et al., 2005) was conducted on barn swallows from New York, USA, male color does not differ in our New York and Colorado populations (Safran, unpublished data).

#### Genetic methods to identify sex in nestlings

Because the role of plumage color as a sexual signal is more clearly defined in male barn swallows (Safran and McGraw, 2004; Safran et al., 2005), we only included males in this study. Male nestlings were identified by adopting the use of polymerase chain reaction (PCR) with sex-linked markers as described by Griffiths et al. (1998) except that 0.25 units of JumpStart *Taq* DNA polymerase (Sigma) was used with a modified thermal cycler protocol: initial denaturation step at 94 °C for 60 s, followed by 34 cycles of 94 °C for 45 s, 48 °C for 45 s, and 72 °C for 45 s, and 72 °C for 3 min for the final extension. PCR products were mixed with SYBR Green (Invitrogen), loaded into a 3% agarose gel in  $1 \times$  TAE buffer, and electrophoresis was used to separate different fragment sizes. The fragments were visualized using a Benchtop UV Transilluminator Digital Imaging System (UVP, LLC); products with one fragment were defined as male and two fragments as female.

#### Statistical analyses

We analyzed the relationship between plumage color and CORT concentration using separate models for adults and nestlings. For all analyses, we used general linear mixed models in SAS (v9.2) with average breast brightness (% reflectance) as the predictor variable (as it relates to the ratio of eumelanin to pheomelanin; McGraw et al., 2005) and, in three separate models either baseline CORT, stress-induced CORT, or stress responsiveness as the response variable. We included only those samples that were collected within 3 min of initial disturbance to represent baseline CORT levels. For adults in our study, there was no significant increase in CORT concentration from blood taken between capture and 3 min (mean  $\pm$  SD:  $1.35 \pm 0.58$  min,  $n = 90$ ,  $F_{1, 85.7} = 3.24$ ,  $P = 0.08$ ). However, nestling baseline CORT concentration was



significantly positively correlated with the amount of time it took to collect the blood sample from initial disturbance (mean  $\pm$  SD:  $1.72 \pm 0.74$  min,  $n = 80$ ,  $F_{1, 39.7} = 26.36$ ,  $P < 0.0001$ ). To correct for a potentially confounding effect of time since capture (up to 3 min) and CORT, we included the variable 'latency time' to indicate the elapsed time from initial disturbance to collection of blood into the nestling statistical models. We also calculated the magnitude of the increase in CORT following the onset of the stressor (stress responsiveness: stress induced CORT minus baseline CORT) to correct for the influence of individual variation in baseline CORT. All CORT measurements (ng/ml) were log-transformed to create a normal distribution. We used JMP software (Professional v.9) to test for collinearity among all predictor variables.

#### Statistical analyses in adults

Barn swallows breed in solitary nesting locations (since they nest at one site) or in colonies where multiple nests are active. To control for non-independence among nests in the same breeding site (colony) we used 'site' as a random effect. Because there are natural changes in both baseline and stress-induced CORT concentrations that coincide with different life history stages within a breeding season (e.g. territory acquisition and nestling provisioning; Romero, 2002; Wingfield and Sapolsky, 2003), the Julian date on which the sample was collected was also included as a random effect. To minimize the effect of circadian changes in circulating CORT on our measurements, blood sampling for adults took place in the evening after birds began to roost on nests. However, because the onset of roosting is partly contingent upon sunset, sampling time occurred later throughout the field season as sunset time advanced, and we found a significant correlation between the time of day that stress-induced blood samples were taken and CORT concentration ( $n = 93$ ,  $F_{1, 87} = 3.01$ ,  $r = 0.31$ ,  $P = 0.003$ ). We therefore used a covariate 'sunset correction' to correct for this potentially confounding effect in our analyses. Mass was significantly negatively correlated with Julian sampling date ( $n = 93$ ,  $F_{1, 88} = 5.16$ ,  $P = 0.03$ ), and was also included with average breast brightness as a covariate in the model.

#### Statistical analyses in nestlings

We analyzed CORT concentrations from a total of 82 male nestlings. In models for nestlings, 'site' was included as a random effect to control for any potentially confounding effects of shared environments on CORT concentrations. The factor 'nest' was included as a random variable in all nestling models to control for any confounding effects of parental influences on CORT levels as well as to control for any repeated observations, which can be due to shared genes (full or half siblings) or shared environment. Julian date of blood collection was also included as a random effect to control for any variation in CORT due to sampling on different days. We included as a fixed effect 'latency time' in the baseline CORT model, which is the time elapsed between initial disturbance and blood collection. There was no significant relationship between time of day that the plasma sample was taken and any of the CORT measurements for nestlings, therefore we did not include time of day in the nestling analyses (mixed linear model with 'nest' and 'sampling date' as random effects: baseline CORT,  $n = 79$ , mass  $F_{1, 61.3} = 8.58$ ,

$P = 0.005$ ; time  $F_{1, 31.3} = 0.42$ ,  $P = 0.52$ ; latency  $F_{1, 44.6} = 25.55$ ,  $P < 0.0001$ ; stress-induced CORT,  $n = 81$ , mass  $F_{1, 66.5} = 1.44$ ,  $P = 0.23$ ; time  $F_{1, 47.2} = 1.90$ ,  $P = 0.1747$ ; swapped  $F_{1, 60.9} = 5.57$ ,  $P = 0.02$ ; stress responsiveness,  $n = 81$ , mass  $F_{1, 68.2} = 0.60$ ,  $P = 0.44$ ; time  $F_{1, 53.2} = 0.14$ ,  $P = 0.71$ ). Full broods were removed from 43 nests, but due to time constraints related to sampling within 3 min of nest removal, we were only able to collect blood samples from a subset of nestlings for some nests (mean total brood size = 3.8, mean collected brood size = 3). Brood size (includes the total number of male and female nestlings within a nest) and mass (g) were included in the model as covariates along with average breast brightness; none of these variables were intercorrelated.

Our data set overlaps with part of a larger study involving a cross-foster experiment where nestlings were switched from their natal nest at day 1 (16 swapped and 66 not swapped). When the category 'swapped' was included as a fixed effect in the model, we found a significant relationship between stress-induced CORT concentration and whether or not a nestling was swapped into a different nest ( $n = 82$ ,  $F_{1, 56.6} = 6$ ,  $P = 0.02$ ); swapped nestlings had lower stress-induced CORT. There was no significant effect of 'swapped' when included as a fixed effect in models with either baseline CORT (mixed linear model:  $n = 80$ , breast brightness  $F_{1, 45} = 0.03$ ,  $P = 0.87$ ; swapped  $F_{1, 33.8} = 0.11$ ,  $P = 0.74$ ; standard baseline time  $F_{1, 27.9} = 0.32$ ,  $P = 0.58$ ; mass  $F_{1, 62.2} = 6.28$ ,  $P = 0.02$ ; brood size  $F_{1, 27} = 0.26$ ,  $P = 0.62$ ; latency  $F_{1, 35.9} = 26.12$ ,  $P < 0.0001$ ), or stress responsiveness (mixed linear model:  $n = 81$ , breast brightness  $F_{1, 69.1} = 0.23$ ,  $P = 0.63$ ; swapped  $F_{1, 59.1} = 3.27$ ,  $P = 0.08$ ; standard baseline time  $F_{1, 47.9} = 0.29$ ,  $P = 0.60$ ; mass  $F_{1, 65.5} = 0.36$ ,  $P = 0.55$ ; brood size  $F_{1, 36.9} = 0$ ,  $P = 0.97$ ) as the response variable, so we excluded 'swapped' from those models and included it as a covariate in our stress-induced model only.

## Results

### Adult males

#### Baseline CORT and plumage color

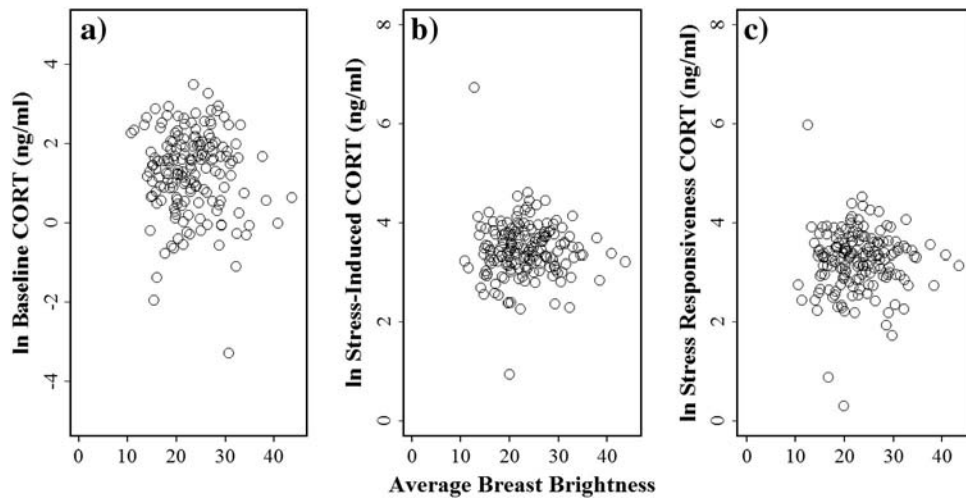
Controlling for variation across breeding sites, Julian date, body mass (g), and sunset correction, we found no statistically significant relationship between male average breast brightness and baseline CORT (mixed linear model:  $n = 90$ , breast brightness  $F_{1, 81.7} = 0.42$ ,  $P = 0.52$ ; mass  $F_{1, 84.8} = 3.35$ ,  $P = 0.07$ ; sunset correction  $F_{1, 84.6} = 3.19$ ,  $P = 0.08$ ). Backwards stepwise model selection revealed a final model that included random effects (breeding site and Julian date) and fixed factors: mass and sunset latency (see Table 1 and Fig. 1a).

#### Stress-induced CORT, stress responsiveness and plumage color

We did not find a significant relationship between breast brightness and stress-induced CORT (see Table 1 and Fig. 1b). There was also no significant relationship between breast brightness and stress responsiveness (see Table 1 and Fig. 1c).

**Table 1**  
Linear mixed models, type 3 sums of squares estimates of adult male CORT profiles as predicted by average breast brightness, mass, and time of day sample was collected corrected by sunset time ('sunset correction'). Site and sampling Julian date were random effects in each model.

Dependent variable	Predictor	Estimate	SE	df	F	P
ln baseline CORT (ng/ml)	Avg breast brightness	-0.01	0.02	1, 81.7	0.42	0.52
	Mass (g)	-0.14	0.08	1, 84.8	3.35	0.07
	Sunset correction	0.26	0.14	1, 84.6	3.19	0.08
ln stress-induced CORT (ng/ml)	Avg breast brightness	-0.002	0.01	1, 84.7	0.04	0.85
	Mass (g)	-0.01	-0.01	1, 84.8	0.13	0.72
	Sunset correction	-0.07	0.08	1, 84.9	0.90	0.35
ln stress responsiveness CORT (ng/ml)	Avg breast brightness	-0.001	0.01	1, 84.5	0.01	0.94
	Mass (g)	0.02	0.04	1, 84.7	0.13	0.72
	Sunset correction	-0.16	0.08	1, 84.9	3.37	0.07



**Fig. 1.** Plumage brightness did not predict circulating CORT in adult male barn swallows a) baseline CORT, b) stress-induced CORT, and c) stress responsiveness (calculated as the difference between baseline and stress-induced CORT). Note that lower brightness values represent darker breast feathers with greater pheomelanin content.

### Nestling males

#### Baseline CORT and plumage color

Similar to the findings in adults, average breast brightness in male nestlings was not predictive of baseline CORT (Fig. 2a) when controlling for site, nest and sampling Julian date as random effects. Heavier nestlings exhibited significantly lower baseline CORT concentrations and baseline CORT concentration was also significantly influenced by latency time (see Table 2 and Fig. 3).

#### Stress-induced CORT, stress-responsiveness and plumage color

We did not detect a statistically significant relationship between nestling breast brightness and either stress-induced CORT or stress responsiveness (Figs. 2b and c). However, nestlings that had been swapped among nests had significantly lower stress-induced CORT concentrations when compared to control nestlings (see Table 2).

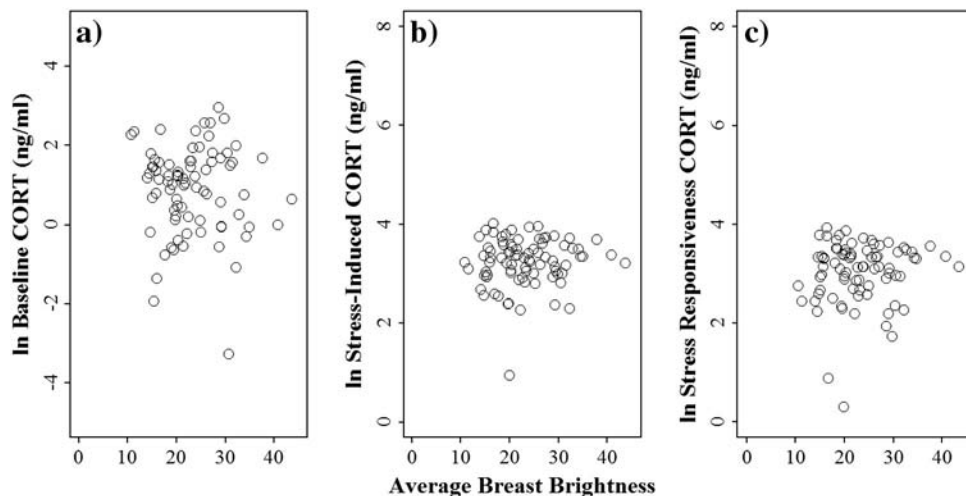
### Discussion

#### Testing the melanin–CORT pleiotropy hypothesis

It has been proposed that melanin-based traits convey information about an individual's ability to cope with stress such that individuals with darker eumelanin signals exhibit greater stress resistance (Ducrest

et al., 2008; Roulin et al., 2008). Based on empirical studies suggesting AGRP, an  $\alpha$ -MSH antagonist, as a potential modulator of both the HPA axis and melanogenesis (Takeuchi et al., 2000; Xiao et al., 2003), we proposed a modified version of the Ducrest et al. (2008) melanocortin hypothesis for pheomelanin-based traits: that darker, more pheomelanin individuals would exhibit greater stress reactivity. Despite known biochemical associations between melanin production and CORT secretion pathways, we found that neither baseline CORT, stress-induced CORT, nor stress responsiveness was predicted by breast brightness in adults during the breeding season when this trait is being advertised, and was similarly unrelated to breast brightness in nestlings when melanin-based plumage is being developed.

Relatively few studies have assessed the relationship between pheomelanin-based traits and natural levels of CORT, and there appears to be greater empirical support in the literature for the role of eumelanin-based traits as honest signals of an individual's ability to dampen the stress response (Almasi et al., 2008, 2010). However, while many studies that have analyzed the relationship between individual variation in melanin coloration and plasma CORT levels focus on plumage patches defined as either “eumelanin” (Almasi et al., 2008, 2010; reviewed in Ducrest et al., 2008) or “pheomelanin” (Almasi et al., 2008; Roulin et al., 2008), most avian plumage is comprised of both of these pigments deposited into the feathers (McGraw, 2006; McGraw et al., 2005). Even though a high proportion of the total melanins in the breast plumage of



**Fig. 2.** Plumage brightness was not associated with CORT levels in nestling male barn swallows a) baseline CORT, b) stress-induced CORT, and c) stress responsiveness.

**Table 2**  
Linear mixed models, type 3 sums of squares estimates of nestling male CORT profiles as predicted by average breast brightness, mass, and brood size. Time from disturbance to sample collection ('latency time') was a fixed variable in the baseline CORT model, and 'swapped' was a fixed variable in the stress-induced CORT model. Site, nest, and sampling Julian date were random effects in each model. Boldface terms indicate statistical significance ( $P < 0.05$ ).

Dependent variable	Predictor	Estimate	SE	df	F	P
ln baseline CORT (ng/ml)	Breast brightness	0.003	0.01	1, 66.8	0.06	0.81
	<b>Mass (g)</b>	<b>-0.14</b>	<b>0.05</b>	<b>1, 48.9</b>	<b>8.05</b>	<b>0.01</b>
	Brood size	0.03	0.09	1, 28.3	0.11	0.74
	<b>Latency time</b>	<b>0.01</b>	<b>0.002</b>	<b>1, 40.0</b>	<b>25.79</b>	<b>&lt;0.0001</b>
ln stress-induced CORT (ng/ml)	Breast brightness	0.005	0.007	1, 67.6	0.42	0.52
	Mass (g)	-0.02	0.02	1, 68.1	0.69	0.41
	Brood size	0.04	0.04	1, 39.3	0.75	0.39
	<b>Swapped</b>	<b>0.30</b>	<b>0.12</b>	<b>1, 56.1</b>	<b>5.93</b>	<b>0.02</b>
ln stress responsiveness CORT (ng/ml)	Breast brightness	0.004	0.01	1, 72.1	0.16	0.69
	Mass (g)	0.02	0.03	1, 68.6	0.52	0.47
	Brood size	-0.02	0.05	1, 36.8	0.15	0.70

male barn swallows are eumelanins, individual variation in the color of this sexually selected trait is determined largely by the percentage of pheomelanins within feathers (McGraw et al., 2005). If the functional link between melanin-based traits and the stress response relies solely on the biochemistry between molecules involved in eumelanogenesis, then the *melanin-CORT pleiotropy hypothesis* may not be suitable for predicting a relationship between color and CORT in systems where pheomelanins are the dominant driver of phenotype variation. Alternatively, because of the high percentage of eumelanins in addition to the importance of pheomelanin content in male barn swallow color variation, this complex trait may not signal information about stress resistance due to the opposing effects that  $\alpha$ -MSH and AGRP may have on the HPA axis and melanogenesis.

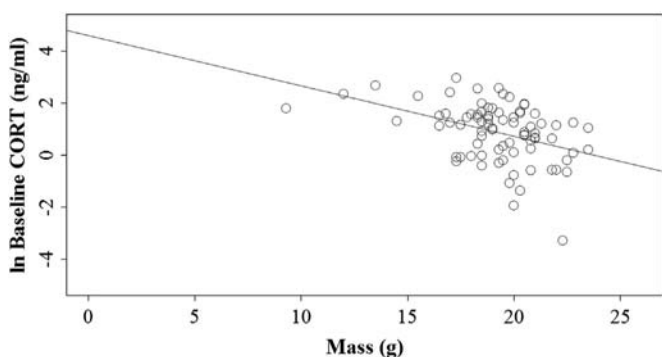
The few empirical studies that have assessed the relationship between CORT and pheomelanogenesis suggest that the biochemical and physiological interactions are often complex and opposing, leading to conflicting predictions on the relationship between the different HPA axis and melanogenesis components. As previously described, there exist physiologically-opposing biochemical pathways that involve  $\alpha$ -MSH and AGRP acting at MCRs in the brain and in the skin to modulate the components of the HPA axis and melanin pigment production, respectively. In addition to regulatory pathways initiated in the brain, regulation of these processes by HPA axis components can also take place at the level of the skin. Studies have shown that CORT is capable of blocking melanogenesis during feather development (Slominski et al., 2004), thus there should be less eumelanin or pheomelanin deposited into feathers for individuals that have heightened stress-induced CORT levels (Almasi et al., 2010; Roulin et al., 2008). Alternatively, CORT could be acting more locally to alter the appearance of the feather, as opposed to blocking melanogenesis, during feather development. Glucocorticoids have been shown to increase protein catabolism and may restrict the amino acids available to feather growth (Hess, 2002; Lattin et al., 2011). Heightened CORT secretion over an extended period of time could change the feather protein composition

and overall feather quality (Galván and Solano, 2009), altering the appearance of color through changes in the feather microstructure while the process of melanogenesis remains intact. Similar to CORT, ACTH is secreted in a circadian rhythm and increases during a stress response, but acts as an agonist to melanocortin receptors in the skin to upregulate melanogenesis (Rousseau et al., 2007); therefore we would expect that individuals with higher ACTH (and, therefore, CORT) levels would produce darker eumelanin pigment. There is a dynamic relationship between the modulation of the HPA axis and melanogenesis where melanocortin agonists and antagonists are proposed to alter HPA axis activity, and the products of the HPA axis (ACTH and CORT) are also shown to elicit effects on melanogenesis at the level of the skin. Therefore, the relationship between color and CORT may also be dynamic throughout the pigment production process.

#### *Temporal variation in development and expression of melanin plumage color*

In birds, there is a small window of time during which plumage is developed and when the melanin pigments in the feathers are actually influenced by the underlying physiology of the bird. In barn swallows, adults molt annually, replacing all contour feathers (including their melanin-based ventral plumage) in the non-breeding season (Brown and Brown, 1999). In order to address the possible differences in physiological state between the time when pigments are being deposited into growing feathers and when plumage color is acting as a sexual signal, we analyzed the relationship between color and CORT in adults (trait advertisement) and nestlings (trait development). Because it is not feasible to locate adults from our breeding population during molt, we used CORT measurements from nestlings as a way to capture any physiological differences present during pigment deposition alongside feather development. As nestling color is predictive of adult color (Hubbard and Safran, unpublished data), an analysis of CORT during nestling plumage development appears to be a relevant measure in lieu of tracking adults during molt in nonbreeding groups in Central and South America. Our results clearly show that breast plumage coloration does not reliably reflect underlying CORT profiles at either the time of trait development in nestlings or trait advertisement in adults.

Due to the nature of feather color being a relatively static trait compared to seasonal fluctuating stress physiology, melanin-based feather traits may honestly signal individual CORT profiles only at certain times of day or in certain environmental (Roulin et al., 2008, 2011) or life history stage (Almasi et al., 2008) contexts. Regulation of the HPA axis by melanin production agonists may differ at different times of day. For example, we know that baseline CORT concentrations vary throughout the day in a circadian fashion, and the changes in pigment production may reflect these changes only at certain time intervals (Roulin et al., 2010). Although there was no relationship between the time of day that plasma samples were taken and any of the CORT measurements for nestlings, we did find a relationship between sampling



**Fig. 3.** Heavier nestlings (g) had lower baseline CORT concentrations compared to nestlings lighter in mass (g).



time and stress-induced CORT in adults where CORT concentration increased as time of day advanced. Future studies could employ the method of extracting CORT deposited into feathers as another potential approach to assessing overall HPA axis activity throughout feather growth (Bortolotti et al., 2008) in conjunction with utilizing feather growth band patterns (Jovani et al., 2011) to better elucidate CORT secretion patterns during feather growth. Because our measurements of baseline and stress-induced CORT samples are taken from one point in time, they may not be the best representation of an individual's overall CORT profile or their 'resistance to stress'. Other ways that 'resistance to stress' has been measured include the time it takes to return to baseline levels after experimentally increasing CORT (Almasi et al., 2010) and changes in behavior such as nestling provisioning rate following exposure to exogenous CORT (Almasi et al., 2008).

Another important aspect of the stress response is how quickly an acute stress response is terminated via negative feedback, which has been shown to predict survival during severe environmental stressors (Romero and Wikelski, 2010). The ability to terminate the stress response can be quantified by administering the synthetic glucocorticoid dexamethasone (DEX), which activates the HPA negative feedback loop and reduces circulating CORT (Sapolsky and Altmann, 1991). Future studies assessing whether melanin-based traits signal an individual's ability to cope with a stressor should take into account these different approaches to measuring stress resistance.

#### CORT and body mass

Our results showed that nestling baseline CORT is significantly negatively correlated with mass, which is in agreement with other CORT studies that have shown a similar relationship between mass and body condition (Angelier et al., 2007; Malisch et al., 2007). That our finding with mass and baseline CORT is consistent with other empirical research provides evidence to support the accuracy of our sample collection regime and statistical approach. Baseline CORT is important in many metabolic processes aimed at maintaining energy reserves, so it is appropriate that mass is negatively associated with baseline CORT. Heightened baseline CORT levels in avian species function to support increased metabolic demands that can occur during the breeding season or in response to decreased food availability, as well as in preparation for migration (Romero, 2002). Given that baseline CORT is a strong predictor of mass and is also functionally linked to maintaining energy levels and energy stores contributing to overall body condition (Landys et al., 2006), it is important to take into account mass when assessing the variation in baseline CORT in relation to other traits.

#### Conclusion

It is clear that there is still a gap in our knowledge of how phaeomelanin-based traits would fit into the melanocortin pleiotropy hypothesis proposed by Ducrest et al. (2008); in particular, the biochemical and physiological interactions between phaeomelanin production and the HPA axis are complex and sometimes opposing. There could be synergistic effects of melanocortin receptor agonists (i.e.  $\alpha$ -MSH and ACTH), antagonists (i.e. ASIP and AGRP), and CORT on the stress axis as well as at the level of the skin to modulate melanogenesis and an individual's stress response simultaneously. Furthermore, the majority of studies analyzing the underlying biochemical basis of HPA axis and melanocortin interactions are with human or mammalian subjects, which may have fundamentally different mechanisms from each other and from avian species. For example, ACTH is locally manufactured in melanocytes and acts as an agonist on MCRs to stimulate eumelanogenesis in humans (e.g. Wakamatsu et al., 1997; Wood et al., 2006), but ACTH is produced at much smaller quantities in rat melanocytes and does not appear to play a role in melanogenesis (Mountjoy, 1994). Given the roles that ACTH and CORT play in melanogenesis are not conserved across taxa, it would be informative for this to

be tested in an avian system (Ling et al., 2004; Takeuchi et al., 2000). Our finding that a predominantly phaeomelanin-based trait does not predict circulating CORT suggests that phaeomelanin and eumelanin signals may convey different physiological information, and highlights the need for further study to tease apart the underlying mechanisms linking the HPA axis and the production of different melanin-based pigments.

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